Effects of Perinatal Exposure to Bisphenol A on Play Behavior of Female and Male Juvenile Rats

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In higher vertebrates, estrogen can exert an organizational effect on sexually dimorphic areas of the central nervous system (CNS) during the perinatal phase of development. The possibility that estrogenic pollutants may mimic estrogen action on the CNS during development and produce long-lasting or irreversible effects is an issue of great concern. Bisphenol A (BPA), a compound widely used in the food industry and in dentistry, has proven estrogenic actions. To study its potential developmental effects on behavior, we gave female Sprague-Dawley rats 40 µg/kg/day BPA from conception to weaning postnatal day 21 and 400 µg/kg/day BPA from gestation day 14 to postnatal day 6. After exposure, we studied social behavior in a play situation in juvenile male and female offspring. The attempt to use play behavior to study the effects of BPA yielded some interesting results. We observed an early action of BPA on several behavioral categories in both males and females. In particular we observed a masculinization of female behavior in two behavioral categories (play with females and sociosexual exploration), an effect probably mediated by the estrogenic activity of BPA in the CNS. These long-lasting effects of BPA could have important consequences at individual and population levels. *Key words:* **bisphenol A, environmental estrogens, play behavior, rat, sex differences, social behavior.** *Environ Health Perspect* **110(suppl 3):403–407 (2002).**
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In higher vertebrates, estrogens exert an organizational effect on the central nervous system (CNS) during the perinatal phase of development, and estrogen is the main hormone responsible for sex differences in behavior (*1,2*). There is great concern that estrogenic pollutants, at low concentrations, may mimic the action of estrogens on the CNS at an early age and produce long-lasting or irreversible effects on behavior (*3–5*). Bisphenol A (BPA) is a compound widely used in the food industry for polycarbonate bottles and linings of cans, as well as in dentistry as a hardener for sealants and for tooth lacquering. It is known to have an estrogenic action (*6–8*). Low doses of BPA administered perinatally can modify explorative behavior and anxiety in rats (*9*) and can advance puberty in female mice (*10*), although some effects are controversial (*11*). These observations indicate the importance of studying the effects of this compound on complex behaviors expressed during puberty.

In mammals, play is considered crucial for the maturation of adult behavior (*12,13*). Many of the behaviors expressed in a play situation are sexually dimorphic and under the control of sex steroids. For example, rough-and-tumble play is controlled by androgens (*13,14*). Immature sexual elements of play represent a developmental process leading to adult sexual behavior; this process is under the perinatal organizational control of estrogens. Therefore, because of its multiplicity in behavior, play is a useful tool for the study of behavioral alterations (*15*).

To study the potential effects of BPA on behavior, we administered it to dams in two treatments: long periods of exposure to a low-dosage regimen, from conception to weaning of their pups, and short periods of exposure to a high-dosage regimen, from gestation day (GD) 14, when the differentiation of many sexual characters begins (*16*), to postnatal day (PND) 6. The two dosages are in the range of human environmental exposure to BPA and are below concentrations generally considered in toxicological studies. The lower concentration for a longer period of time can be encountered in human food; the higher concentration for a shorter period of time in human saliva after dental interventions (*17,18*). We then studied the behavior of the offspring of the treated mothers in a play situation and considered all behaviors expressed.

Materials and Methods

Subjects

We used 84 immature Sprague-Dawley rats (42 females and 42 males) that were born and bred in the Department of Animal Biology, University of Florence (Florence, Italy), and were the offspring of mothers treated during gestation and lactation, as described below. The subjects were separated into three groups on the basis of their exposure to BPA during the perinatal period: *a*) high-dose treatment, 15 males and 15 females offspring of mothers treated with short periods of exposure to a high dosage of BPA; *b*) low-dose treatment, 12 male and 12 female offspring of mothers treated with long periods of exposure to a low dosage of BPA; *c*) control, 15 female and 15 male offspring of vehicle-treated mothers. The litters were weaned on PND 21. For each treatment group, the animals were randomly chosen from different litters and housed in groups of three males and three females in polysulfone cages, 42 × 26 × 15 cm (Tecniplast, Italy), under a natural light–dark cycle. No cage contained siblings. Food and water were available *ad libitum*.

Treatment Procedure

Bisphenol A (Fluka Ltd., Buchs, Switzerland) dissolved in arachis oil was administered daily at two dosages (40 and 400 µg/kg body weight) to two groups of mothers during pregnancy and lactation.

Thirty-one female rats of reproductive age were randomly allocated to three groups and subjected to the following treatments: *a*) low dose (n = 11), receiving 40 µg/kg/day BPA, administered from day 10 before mating with a mature male until weaning of the pups (PND 21); *b*) high dose (n = 11), receiving arachis oil from day 10 before mating until GD 13, followed by 400 µg/kg BPA from GD 14 (± 1 day) to PND 6, and then arachis oil again until weaning; *c*) control (n = 9), receiving arachis oil from day 10 before mating until weaning. BPA was dissolved in arachis oil at 5.32 and 53.2 µg/mL, for the low-dose and high-dose modalities. Controls received a comparable amount of oil, according to body weight. BPA was administered orally with a pipette. Because the animals enjoyed receiving the oil, the procedure was not stressful. For all females, mating took place 10 days after the beginning of treatment: they were housed three

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per cage for 48 hr with a sexually mature male and then transferred to single cages. The litters were culled to eight at birth and weaned at PND 21. For each treatment group, the pups were randomized and housed in groups of three males and three females such that no cage contained siblings.

Behavioral Testing

Behavioral observations were conducted at PNDs 35, 45, and 55 between 1500 and 1900 under artificial dim white light. Rats belonging to the same cage were tested together for each age, and subjects were individually marked with cosmetic dye.

Table 1. List of social and nonsocial behaviors considered.

Nonsocial behaviors
Air-smelling
Exploration of the environment
Rearing (animal stands up and explores the environment)
Ground exploration
Digging
Self-grooming
Crouching
Social behaviors
Approaching (moving toward another)
Crawl-over (moving over another)
Crawl-under (moving under another)
Social investigation (sniffing another's body except anogenital area)
Anogenital sniffing
Allogrooming (gentle grooming of another's fur)
Aggressive grooming (vigorous grooming of another)
Pouncing (bouncing over another)
Charging (rushing toward another with vigorous bouncing gait)
Chasing
Riding (forepaws over the back of a moving partner)
Sideways posture (the animal orientates itself broadside on to another)
Aggressive posture (the animal orientates itself at right angle to and over another)
Submissive posture (lying on the back with belly exposed to another)
Biting
Withdrawing (all movements away from another)
Upright posture (with erect posture the rat exposes its belly to another)
Boxing (both rats stand up facing each other and boxing with forepaws)
Jumping (animal leaps vigorously into the air)

Behavioral observations took place in a neutral arena (36 × 61 × 34 cm) with the floor covered with clean sawdust. The six cagemates were transferred directly to the arena from the home cage and video-recorded with a Sony Hi8 video recorder for 6 min, starting after 1 min of familiarization. The video recordings were then analyzed by one observer blind to treatment, using Noldus Observer in combination with Noldus Video Tape Analysis System (Version 3.0; Noldus Information Technology, The Netherlands). All behaviors were recorded and the actor and receiver identified for social interactions. For the purposes of the present research, only the frequency of behaviors displayed during min 2 and min 3 of each session were considered.

Play, defined as "any activity involving exaggerated movements and inhibited attacks; it appears to achieve no obvious goal," was identified according to the description of Poole and Fish (*19*), while those behaviors similar to adult behavior were identified according to the description of Grant and Mackintosh (*20*). Table 1 lists the behaviors we considered.

Statistical Analysis

Principal component analysis (PCA) (*21*), with varimax rotation and Kaiser normalization (SPSS software), was performed on frequencies of behaviors of all experimental subjects. Individual factor scores of each principal factor were subsequently used as independent variables in a three-way analysis of variance (ANOVA) considering treatment, sex, and age.

When appropriate, post hoc analysis (Fisher least significant difference test) separately in males and females for comparison of the treatment groups.

Animal Welfare

Experimental procedures followed the regulations of the European Communities Council Directive 86/609/EEC (*22*).

Table 2. Results of PCA applied to behaviors of immature rats: rotated component matrix (varimax) (total variance explained: 69.3%).

	Factor 1 (12.9%)	Factor 2 (11.0%)	Factor 3 (9.0%)	Factor 4 (8.7%)	Factor 5 (7.4%)	Factor 6 (7.3%)	Factor 7 (7.0%)	Factor 8 (6.0%)
Nape–m	0.80	—	—	—	—	—	—	—
Pounce–m	0.79	—	—	—	—	—	—	—
Crawl over–m	0.73	—	—	—	—	—	—	—
Riding–m	0.73	—	—	—	—	—	—	—
Pounce–f	—	0.81	—	—	—	—	—	—
Chase–f	—	0.74	—	—	—	—	—	—
Nape–f	—	0.71	—	—	—	—	—	—
Withdraw–f	—	0.44	—	—	—	—	—	—
Air smelling	—	—	0.88	—	—	—	—	—
Rearing	—	—	0.84	—	—	—	—	—
Sideways posture–m	—	—	—	0.80	—	—	—	—
Withdraw–m	—	—	—	0.79	—	—	—	—
Crawl under–f	—	—	—	—	0.73	—	—	—
Crawl under–m	—	—	—	—	0.57	—	—	—
Anogenital sniffing–f	—	—	—	—	—	0.70	—	—
Social investigation–f	—	—	—	—	—	0.67	—	—
Self–grooming	—	—	—	—	—	0.49	—	—
Digging	—	—	—	—	—	—	0.77	—
Ground exploration	—	—	—	—	—	—	0.63	—
Approach–m	—	—	—	—	—	—	—	0.82
Approach–f	—	—	—	—	—	—	—	0.55

Abbreviations: f, female directed; m, male directed.
Only largest correlation coefficients of each behavior are reported. Percentage of total variance accounted for by each factor is given in parentheses.

Table 3. Three-way ANOVA applied to factor scores.

	Treatment (df = 2, 234)		Sex (df = 1, 234)		Age (df = 2, 234)		Treatment × sex (df = 2, 234)		Treatment × age (df = 4, 234)		Sex × age (df = 2, 234)		Treatment × sex × age (df = 4, 234)	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Factor 1	1.13	NS	0.57	NS	9.28	<0.000	1.15	NS	1.26	NS	2.63	<0.1	0.27	NS
Factor 2	3.46	<0.03	14.80	<0.000	5.88	<0.003	0.17	NS	0.70	NS	0.86	NS	0.64	NS
Factor 3	2.13	NS	0.07	NS	9.23	<0.000	0.99	NS	1.60	NS	0.82	NS	0.48	NS
Factor 4	2.09	NS	1.09	NS	9.02	<0.000	1.88	NS	1.72	NS	0.19	NS	0.88	NS
Factor 5	5.15	<0.01	4.69	<0.05	5.00	<0.007	0.07	NS	2.89	<0.02	0.58	NS	1.56	NS
Factor 6	7.21	<0.001	34.31	<0.000	8.43	<0.000	2.60	<0.1	1.27	NS	4.93	<0.01	0.76	NS
Factor 7	0.74	NS	14.76	<0.000	0.28	NS	1.96	NS	0.47	NS	2.09	NS	0.26	NS
Factor 8	14.69	<0.000	3.24	<0.1	11.30	<0.000	0.53	NS	2.46	<0.05	2.57	<0.1	0.89	NS

Abbreviations: df; degrees of freedom; NS, not significant, $p > 0.1$.

Results

Principal component analysis, excluding infrequent behaviors, shows eight principal factors (Table 2), explaining 69.3% of the variance. Factor 1 groups play behaviors directed to males; factor 2, play behaviors directed to females; factor 3, nonsocial exploration; factor 4, defensive behavior; factor 5, low-intensity mating elements; factor 6, sociosexual exploration; factor 7, ground exploration; factor 8, social interest. Three-way ANOVA conducted for each factor, using individual factor scores as variables, reveals significant effects of treatment, sex, and age. BPA administration can significantly modify behaviors grouped under factors 2, 5, 6, and 8 (Table 3); this is generally true for both the high-dose and low-dose modalities (Table 4). Significant sex differences are evident for factors 2, 5, 6, and 7 (Table 3). As expected, during this period of development, the effects of age are pervasive and significantly affect all but factor 7 (ground exploration). Factors 5 and 8 yield a significant treatment × age interaction. Sex × treatment interaction is not significant.

To have a general view of the effects of treatment on each factor, we pooled the three age groups and performed statistical comparisons on mean factor scores (Table 4). To better understand and visualize the magnitude and direction of the effect, we drew graphs for each factor, considering the total frequency of the behaviors with a factor loading > 0.5.

The frequency of play behavior directed to females (grouped under factor 2) was increased by BPA—the low-dose treatment being more effective in females and the high-dose treatment being more effective in males (although not significantly) (Figure 1, Table 4). The frequency of crawl-under behavior in females and males, grouped under factor 5 (low-intensity mating elements), was decreased by the high-dose and low-dose modes in both males and females (Figure 2, Table 4). The frequency of sociosexual exploration (genital sniffing and body sniffing, grouped under factor 6) was decreased by both modalities in males, whereas the two modalities produced opposite effects in females (Figure 3, Table 4). The frequency of social interest (approach to females and approach to males, grouped under factor 8) was decreased in both sexes by the high-dose modality (Figure 4, Table 4). We evaluated by one-way ANOVA the effect of treatment on behavior around the critical age of vaginal opening, considering subjects at PND 35 to be of age. A significant increase of social interest (factor 8) is evident in females ($F = 5.35$, $p < 0.01$, df = 2, 39) (Figure 5) and in males ($F = 4.48$, $p < 0.02$, df = 2, 39) (Figure 6) with the low-dose modality. Crawl-under behavior (factor 5) is significantly depressed in females ($F = 3.76$, $p < 0.03$, df = 2, 39) (Figure 5) and marginally in males ($p < 0.01$) (Figure 6) by both treatments. In males, we observed a significant decrease in sociosexual exploration (factor 6) due to both treatments ($F = 3.34$, $p < 0.05$, df = 2, 39) (Figure 6).

Table 4. Post hoc comparisons (Fisher least significant difference) between treatment groups of males and females based on mean factor scores.

	Females			Males		
	Control ($n = 45$)	Low dose ($n = 45$)	High dose ($n = 36$)	Control ($n = 45$)	Low dose ($n = 45$)	High dose ($n = 36$)
Factor 1	–0.04 ± 0.12	–0.04 ± 0.09	–0.05 ± 0.13	0.23 ± 0.20	–0.19 ± 0.15	0.10 ± 0.20
Factor 2	–0.41 ± 0.07	–0.08 ± 0.15*	–0.19 ± 0.07	–0.01 ± 0.14	0.35 ± 0.16	0.38 ± 0.26
Factor 3	–0.23 ± 0.13	0.25 ± 0.17	–0.07 ± 0.16	–0.06 ± 0.18	0.05 ± 0.11	0.07 ± 0.16
Factor 4	0.26 ± 0.18	–0.11 ± 0.09	0.06 ± 0.15	–0.17 ± 0.13	–0.21 ± 0.13	0.22 ± 0.23
Factor 5	0.08 ± 0.17	–0.12 ± 0.16	–0.40 ± 0.09 *	0.30 ± 0.19	0.20 ± 0.13	–0.17 ± 0.13*
Factor 6	–0.23 ± 0.09	–0.21 ± 0.10	–0.59 ± 0.08*#	0.73 ± 0.19	0.15 ± 0.15*	0.04 ± 0.20*
Factor 7	0.14 ± 0.15	0.33 ± 0.15	0.23 ± 0.16	0.00 ± 0.18	–0.28 ± 0.12	–0.46 ± 0.12
Factor 8	0.23 ± 0.14	0.35 ± 0.20	–0.36 ± 0.13*#	–0.12 ± 0.12	0.27 ± 0.15*	–0.55 ± 0.11*#

*$p < 0.05$ versus control group; #$p < 0.05$ versus other BPA treatment.

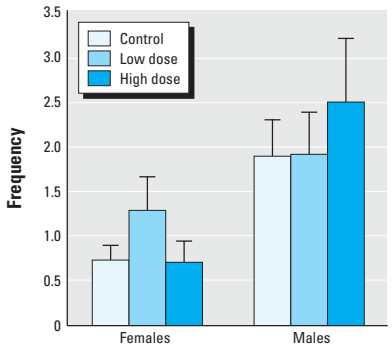

Figure 1. Effects of two different modalities of BPA administration on play directed toward females. Factor 2: pouncing, female; chasing, female; nape, female. Graphs are calculated on the frequencies of behavioral components with loading > 0.5 (mean ± SE). See Table 4 for statistical comparisons among mean factor scores.

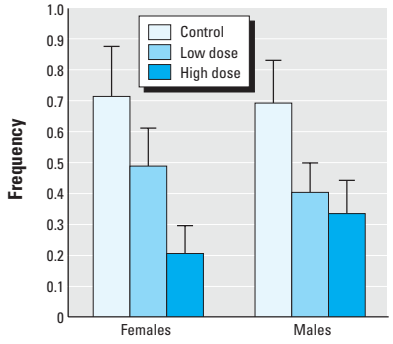

Figure 2. Effects of two different modalities of BPA administration on low-intensity mating elements. Factor 5: crawl-under, both male and female. Graphs are calculated on the frequencies of major behavioral components (mean ± SE). See Table 4 for statistical comparisons among mean factor scores.

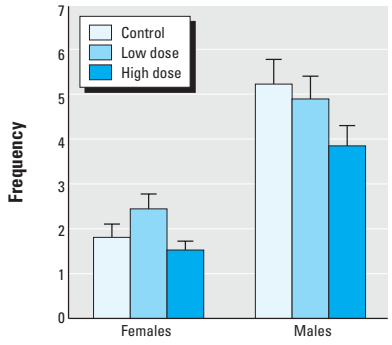

Figure 3. Effects of two different modalities of BPA administration on sociosexual exploration. Factor 6: anogenital investigation, female; social investigation, female. Graphs are calculated on the frequencies of behavioral components with loading > 0.5 (mean ± SE). See Table 4 for statistical comparisons among mean factor scores.

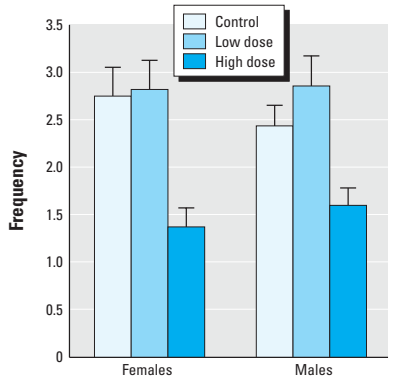

Figure 4. Effects of two different modalities of BPA administration on social interest. Factor 8: approach, both male and female. Graphs are calculated on the frequencies of behavioral components with loading > 0.5 (mean ± SE). See Table 4 for statistical comparisons among mean factor scores.

Discussion

Principal component analysis allowed us to organize the different behavioral elements in a play situation in immature rats under coherent factors, indicating eight general categories of behavior (Table 2): play with males (factor 1), play with females (factor 2),

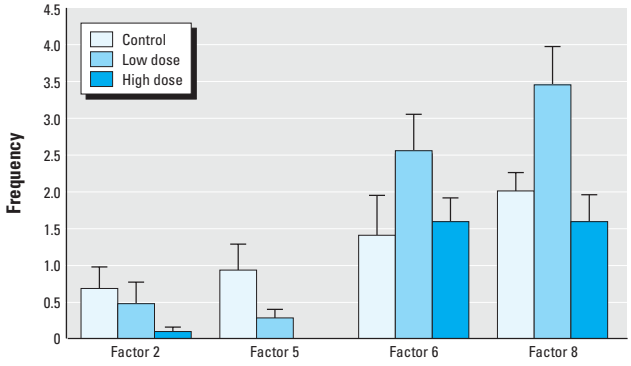

Figure 5. Effects of two different modalities of BPA administration on immature behavior of PND-35 female rats. Graphs are calculated on the frequencies of behavioral components with loading > 0.5 (mean ± SE).

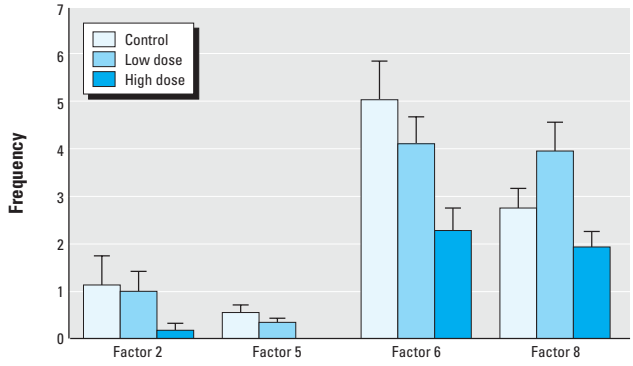

Figure 6. Effects of two different modalities of BPA administration on immature behavior of PND-35 male rats. Graphs are calculated on the frequencies of behavioral components with loading > 0.5 (mean ± SE).

nonsocial exploration (factor 3), defensive behavior (factor 4), low-intensity mating elements (factor 5), sociosexual exploration (factor 6), ground exploration (factor 7), and social interest (factor 8). The subsequent ANOVA using single factors as variables revealed significant effects of BPA treatment (Table 3). Clear sexual differences and effects of age were evident. Both results were expected and they provide a good starting point to study the action of a potential endocrine disruptor. We found significant effects of BPA on the behavior of juvenile rats of both sexes, including social, mild sexual and explorative behavior. Both modalities of perinatal administration—high dose and low dose—proved to be effective.

PCA analysis split elements of rough-and-tumble play into two different factors (1 and 2). This is interesting because rough-and-tumble play is considered to be mediated by nonaromatizable androgens (*13,14*). In contrast, factors 1 and 2 responded differently to BPA administration, revealing an estrogenic action of BPA on factor 2. Play behavior with males (factor 1), which includes elements of rough-and-tumble play, particularly when males interact with males, was far more frequent in control males than in control females. Play behavior with females (factor 2) was, as expected, more frequent in control males than in control females (Figure 1, Table 4). It was increased in females by the low-dose modality and in males by the high-dose modality, although not significantly. The effect of BPA on this factor appears to be a slight masculinization in both sexes, as expected if BPA has an early estrogen-like action.

Factors 5, 6, and 8 are more homogeneous in their components, each including fewer or single behaviors. Factor 6 includes female genital and body sniffing and apparently combines social and sexual interest. These behaviors were more frequent in control males than in control females (Figure 3, Table 3). We observed an increase of this behavior in females after low-dose BPA treatment, suggesting a slight masculinization of females. In contrast, these behaviors were decreased in males by high-dose BPA administration, suggesting a demasculinization. Both factors 2 and 6, which include elements directed to females and are sexually differentiated in controls, are masculinized by BPA in females.

A different pattern has been found for factors 5 and 8. The behaviors under these factors are directed to both sexes and are performed in control females and control males at a similar frequency (Figures 2 and 4, Table 4). Factor 5 includes crawl-under behavior in females and males, which we interpret, after Grant and Mackintosh (*20*), as low-intensity mating elements. The frequency of this behavior is significantly reduced in both sexes after BPA. This could be interpreted as a lowering of sexual interest (but not of sexual orientation, because treated males and females interacted with both sexes with similar frequencies). However, we believe that at a prepubertal age, other components of this behavior could be important (e.g., affiliative and submissive).

Factor 8 includes only approach behavior, directed to both males and females, and we interpret it as general social orientation because it precedes a variety of interactive behaviors. The frequency of this behavior, similarly to factor 5, was decreased by BPA in both sexes, high-dose treatment being more effective than low dose. The strongest effect of BPA treatment was on approach ($p < 0.0001$). However, because of the broad meaning of this behavior and the lack of a sex difference in controls, we are unable to explain this effect in terms of sociosexual behavior.

In general, differences between the high-dose and low-dose modalities were not clear-cut or systematic. The two modalities differed in dosage, duration of treatment, and temporal window of developmental sensitivity. More pronounced effects seem related to the high-dose modality, corresponding also to a shorter period of administration. However, we found the effect of masculinization of females for factors 2 and 6 with the low-dose modality. This could be due to the longer period of treatment, because it is possible that the two sexes have different temporal windows of sensitivity to the substance and that a longer treatment can compensate for the lower dosage.

An interesting study by Howdeshell et al. (*10*) found an advancement of puberty in female mice treated prenatally with BPA. In the present study, we found an increase, compared with controls, at PND 35 in females for social interest (factor 8; low-dose modality), a behavior that includes interaction with males and females (Figure 5); in contrast, low-intensity mating elements (factor 5) are depressed by both treatments (Figure 5). These results are not contrary to those of Howdeshell et al. (*10*): we do not know how first estrus and these behaviors are related. Recent results from our laboratories show in females rats treated perinatally with BPA a significant advancement of the first estrus (*23*). Males at PND 35 show a similar increase due to treatment (low-dose modality) for social interest (factor 8) and a decrease for behaviors under factors 2, 5, and 6 (significant for factor 6) (Figure 6). The alteration of the pace of maturation is not homogeneous in different behavioral categories, suggesting the possibility that these behaviors are under the control of different neuroendocrine subsystems.

In summary, in controls we observed sex differences in four behavior categories (play with females, low-intensity sexual behavior, sociosexual exploration, and ground exploration), and in groups treated with two different doses of BPA, we observed a masculinization of female behavior in two of these categories (play with females and socio-

sexual exploration). Moreover, in one category (play with females), we observed an intensification of male behavior in males due to BPA. Both BPA doses were below concentrations generally considered in toxicologic studies. Our results appear to agree with the hypothesis of an early action of BPA mediated by its estrogenic activity at the CNS.

Conclusions

We have attempted to use a complex behavior model—play behavior—to study the effects of potential endocrine disruptors on behaviors controlled by sex steroids during development. Using this approach, we observed many significant effects of the environmental estrogen BPA. The action of BPA was long-lasting. Our research suggests that very important mechanisms underlying certain behaviors are involved, and thus, in the long run, even small changes can have consequences on individual fitness and on population structure. The mechanisms underlying the observed effects need to be clarified by further research.

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